VIP Ash Dieback Very Important Paper

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# A Volatile Lactone of *Hymenoscyphus pseudoalbidus*, Pathogen of European Ash Dieback, Inhibits Host Germination**

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***Abstract:*** *The largely unknown secondary metabolism of the plant pathogenic fungus Hymenoscyphus pseudoalbidus was investigated by use of the CLSA method. A set of volatile lactones was identified by GC/MS. The lactones were synthesized and used in bioassays in which one of the compounds was found to be a strong germination inhibitor for ash seeds, causing necroses in the plant tissue.*

The common or European ash, *Fraxinus excelsior*, is an ecologically and economically important tree that is native to most of Europe and parts of Asia near the Black Sea. The tree is being threatened by ash dieback, a disease caused by the pathogenic fungus *Hymenoscyphus pseudoalbidus*, an apparently invasive species native to eastern Asia.[1] In the course of the last two decades, the disease has been rapidly spreading from east to west across the European continent, in 2012 crossing the English channel.[2–11] The known phytotoxin viridiol was recently suggested as an important virulence factor of *H. pseudoalbidus*.[12] However, since this compound is also produced by the avirulent sister species, *H. albidus*, and concentrations of the metabolite in different isolates do not correlate with virulence, viridiol cannot be the only virulence factor responsible for pathogenicity.[13] The pathogen produces exoenzymes required for degradation of the host tissues,[13] while other phytotoxins have not been reported. Here we show that the volatile lactone 3,4-dimethylpentan-4-olide, which is emitted as a mixture of enantiomers by *H. pseudoalbidus*, strongly inhibits seed germination of *F. excelsior* and causes necrotic lesions in plant tissues. The strongest inhibitory effect was observed for the racemic compound, while both pure enantiomers were slightly less active. Our results show that 3,4-dimethylpentan-4-olide is a phytotoxin of *H. pseudoalbidus* and may play an important role as a virulence factor in ash dieback. Knowledge of this phytotoxin is important because it lays the ground for future investigations to find its molecular target and thus for understanding pathogenicity of *H. pseudoalbidus* and resistance mechanisms to the pathogen in ash. *H. pseudoalbidus* (syn. "*Chalara fraxinea*") is the causal agent of ash dieback, a disease that in the course of the last two decades has been increasingly threatening the entire population of the common ash (*F. excelsior*) in Europe.[2–4] Recently, ash dieback resulting from *H. pseudoalbidus* has also been found in other ash species, including the narrow-leafed ash (*Fraxinus angustifolia*).[14] Upon infection, the fungus causes wilting of leaves, dieback of the crown, necrotic lesions in the bark, and a discoloration of the wood.[15] A small, but evolutionary important fraction of the ash population seems to be resistant to the fungus, but it is unclear whether this portion is large enough to prevent the acute collapse of ash forests and to ensure survival of the species.[16] The pathogen, *H. pseudoalbidus*, and its avirulent sister species, *H. albidus*, are morphologically almost identical, but can be differentiated at the DNA level. Epidemiological studies,[17] as well as investigations of herbarium specimens, suggest that *H. albidus* is slowly replaced by *H. pseudoalbidus*,[18] though the evolutionary advantage of the pathogen remains unclear. Only little is known about the secondary metabolism of *H. pseudoalbidus*, knowledge of which may offer a key to understanding its pathogenicity. The characterized secondary metabolites that were isolated from culture extracts of *H. pseudoalbidus* include viridiol (**1**, Figure 1), viridin (**2**), and several viridin-like furanosteroids.[12,13,19] Whereas viridiol was shown to be phytotoxic to *F. excelsior* in leaf segment tests,[12] later investigations demonstrated that the concentrations of this metabolite in culture extracts of the individual *H. pseudoalbidus* isolates do not correlate with their respective and variable virulence.[13]

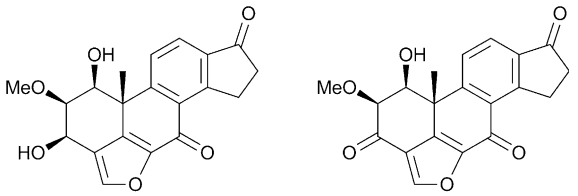


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***Figure 1.*** Structures of the known phytotoxins viridiol (**1**) and viridin (**2**).

Thus, other secondary metabolites may be more important for mediating virulence of the pathogen. Only the full chemical characterization of yet unidentified secondary

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metabolites from *H. pseudoalbidus* and their subsequent large-scale fermentations or synthesis will make it possible to assay their roles in the plant–pathogen interaction. This, in turn, will enable research to focus on control of this pathogen.[3,4] We have recently started a dual approach in analyzing non-volatile secondary metabolites from liquid culture extracts and the volatiles from headspace extracts. An interesting finding was the identification of hymenosetin, a new 3-decalinoyltetramic acid antibiotic, from *H. pseudoalbidus*,[20] though this compound does not seem to play a role in pathogenicity. Here we report on the identification, synthesis and bioactivity of a series of volatile lactones that are released by *H. pseudoalbidus* and that are toxic to *F. excelsior*.

In order to investigate the profile of volatile secondary metabolites emitted by *H. pseudoalbidus*, agar plate cultures of four strains of *H. pseudoalbidus* (C494, C498, C505, and C506) that had been isolated from infected ash trees from a nursery in Ellerhoop (Germany) were analyzed by use of a closed-loop stripping apparatus (CLSA) in combination with GC/MS.[21,22] The method makes use of a circulating air stream in a closed system that is directed over a biological sample such as a petri dish with a microbial culture and then through a charcoal trap for capturing the volatile material released by the sample. After elution with a solvent, a concentrated headspace extract is obtained that can be directly analyzed by GC/MS. The virulence of all four strains was confirmed in pathogenicity tests by infection of axenic seedlings of *F. excelsior* (Table 1).[13]

***Table 1:*** Pathogenicity tests with *H. albidus* and *H. pseudoalbidus*.

| Isolate | Asymptomatic[a] [%] | Symptomatic[a] [%] | Dead[a] |
|---|---|---|---|
| Control | 100 | 0 | 0 |
| C494 | 25.0 | 62.5 | 12.5 |
| C498 | 0 | 50.0 | 50.0 |
| C505 | 37.5 | 50.0 | 12.5 |
| C506 | 12.5 | 75.0 | 12.5 |
| CAR1 | 100 | 0 | 0 |

[a] In each experiment eight individual axenic seedlings of *F. excelsior* were infected with a strain of *H. pseudoalbidus* (C494–C506) or with *H. albidus* (CAR1). No infection was performed in the control experiment. The percentages of asymptomatic, symptomatic (necrotic lesions), and dead seedlings are given.

For comparison, an isolate from the Camargue region in France of the avirulent species *H. albidus* (CAR1) was also included. Representative chromatograms obtained from all five strains are shown in Figure 2. The bouquets were dominated by five compounds for which the structures of the methyl ester **3** and the lactones **4**–**7** were suggested from their mass spectra (see Figure S1 in the Supporting Information). The structures were confirmed by comparison with synthetic references (Supporting Information).[23–25] The same set of compounds was identified in 12 of the 16 other *H. pseudoalbidus* isolates, while four isolates did not produce these volatiles (Supporting Information).

With the synthesized compounds in hand, an initial bioassay was performed to investigate their bioactivity against *F. excelsior*. Ash seeds were placed onto an agar growth

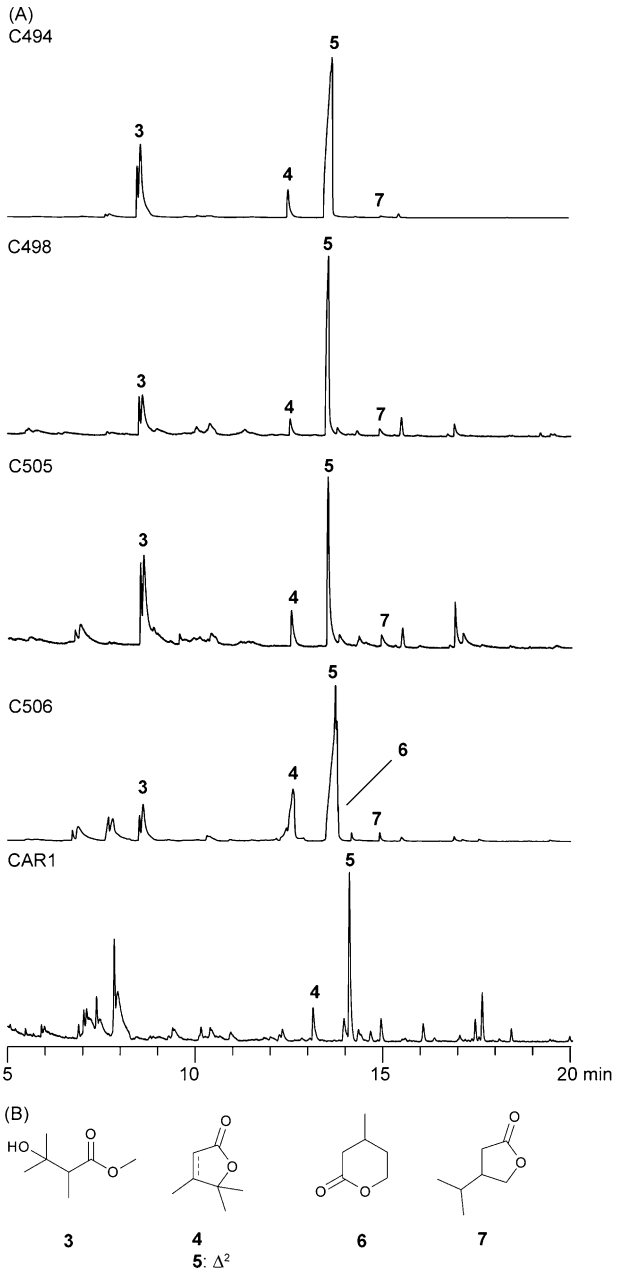



***Figure 2.*** Identification of volatiles from *H. pseudoalbidus* and *H. albidus*: A) Total ion chromatograms of CLSA headspace extracts from five individual isolates (*H. pseudoalbidus* strains C494, C498, C505, and C506, and *H. albidus* CAR1); B) structures of volatiles.

medium, containing 50 $\mu g\,mL^{-1}$ of each tested substance, and incubated for two weeks. Germination was completely inhibited by 3,4-dimethylpentan-4-olide (**4**), while the other compounds showed minimal to no activity (Figure 3). A close-up view of the *F. excelsior* seeds in the bioassay with **4** showed that this lactone also caused necrotic lesions on the seeds that were not observed in the solvent control and the germination inhibition tests with the other volatiles from *H. pseudoalbidus* (Figure 4). For detailed investigations on the bioactivity of **4**, a synthetic route to both enantiomers was developed starting from the commercially available Roche esters (*S*)- and (*R*)-**8** (Scheme 1). The Roche esters were converted into the diols **9**

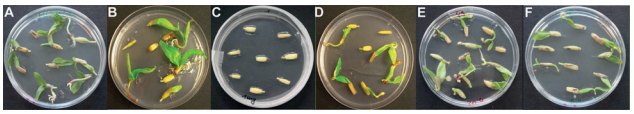
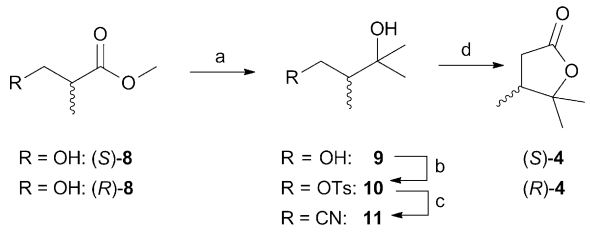



***Figure 3.*** Germination assay with volatiles from *H. pseudoalbidus* against seeds of *F. excelsior*. A) Control sample with solvent; B) (*rac*)-methyl 3-hydroxy-2,3-dimethylbutanoate (**3**); C) (*rac*)-3,4-dimethylpentan-4-olide (**4**); D) 3,4-dimethylpent-2-en-4-olide (**5**); E) (*rac*)-3-methylpentan-5-olide (**6**); F) (*rac*)-3-(1-methylethyl)butan-4-olide (**7**). All compounds were tested at a concentration of 50 $\mu g\,mL^{-1}$.


***Figure 4.*** Necroses on ash seedlings upon treatment with **4**. A) Solvent control; B) whole seedling and C) close-up view of necroses that developed in the germination assay with lactone **4**.




***Scheme 1.*** Synthesis of both enantiomers of **4** starting from the (*S*) and (*R*) Roche esters: a) MeMgBr, 85%; b) TsCl, pyridine, 68%; c) KCN, DMSO, 80°C, 79%; d) NaOH, $H_2O$/MeOH, then HCl, 90%. Ts = tosyl, DMSO = dimethylsulfoxide.

by Grignard reaction with methylmagnesium bromide, followed by treatment with tosyl chloride in pyridine to give the tosylates **10**.[26] Nucleophilic substitution with potassium cyanide in hot dimethyl sulfoxide resulted in the cyanides **11**, which provided the enantiopure lactones (*S*)- and (*R*)-**4** (>99% *ee*) upon saponification and cyclization with 41% overall yield. GC analysis of racemic **4**, the pure enantiomers, and the CLSA headspace extracts from *H. pseudoalbidus* C506 on a chiral stationary phase showed that the natural composition of enantiomers is a 1:10 mixture of (*S*)-**4**/(*R*)-**4**, equal to 80% *ee* (Figure 5).

The same enantiomeric composition was observed for the *H. pseudoalbidus* strain C494. To further investigate the potency of **4** in the germination inhibition assay, the racemic compound and both pure enantiomers were tested on ash seeds in concentrations ranging from 1 mM to 1 nM (Figure 6).

Generally, germination inhibition was found to be strongest for (*rac*)-**4**, followed by (*R*)-**4**, the major enantiomer of the natural product, while the (*S*) enantiomer showed lowest activity. Furthermore, a clearly concentration-dependent activity of **4** was found to exhibit a strong effect in the range of 1 mM to 1 μM in all activity tests with the racemate and both pure enantiomers. At lower concentrations between 100 nM and 1 nM, only the racemate and pure (*R*)-**4** inhibited

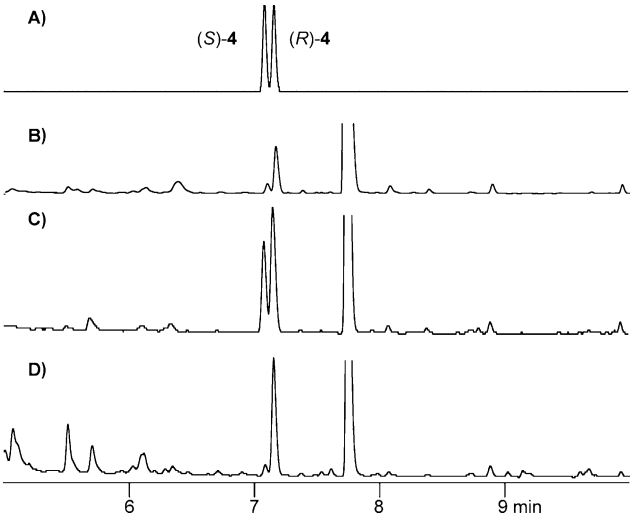



***Figure 5.*** Determination of the absolute configuration of **4** by GC on a chiral stationary phase. Analysis of A) (*rac*)-**4**, B) headspace extract from *H. pseudoalbidus* C506, C) mixture of the natural sample with (*rac*)-**4**, and D) mixture of the natural sample with (*R*)-**4**.

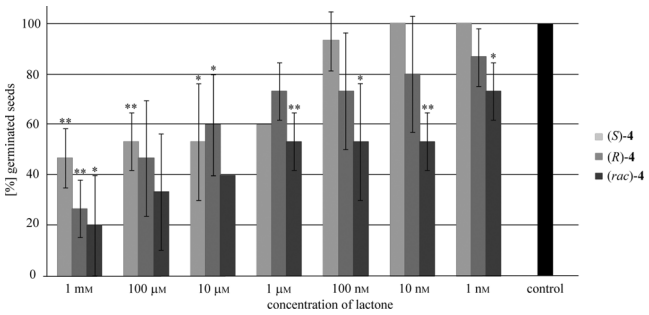



***Figure 6.*** Germination assay with both enantiomers and the racemic mixture of **4** against *F. excelsior* seeds. Values are means of three replicates ± standard deviation. Asterisks indicate *p* values: ** $p \le 0.01$; * $p \le 0.05$.

germination, whereas the (*S*)-enantiomer was inactive. For comparison, the germination inhibitory activity of **4** was also tested against *Ocimum basilicum*, a member of the same plant order to which *F. excelsior* belongs (Lamiales), resulting in no detraction of seed development and suggesting that **4** interacts with a specific target that is restricted to *F. excelsior*.

The germination inhibitory effect of **4** is contrary to the well-known promotion of seed germination by karrikins, a class of butenolides that are present in plant-derived smoke.[27,28] Although the molecular target of **4** is unclear, this lactone is likely a major contributor to the pathogenicity of *H. pseudoalbidus* toward ash. However, other pathogenicity factors may also be relevant, because **4** could not be detected in some pathogenic *H. pseudoalbidus* isolates.

Furthermore, the fact that **4** is also produced by a non-pathogenic *H. albidus* control strain initially seems to speak against its role as virulence factor, though its bioactivity in terms of inhibition of germination and generation of necrotic lesions in plant tissues was clearly demonstrated. An explanation of these findings could be a differential regulation of the

production of **4** in infected plants, possibly by a secondary metabolite or similar factor from *F. excelsior*. This would mean that the biosynthetic potential of the virulence factor **4** is present in both *H. albidus* and *H. pseudoalbidus*, but the pathway is only activated in the invasive pathogen, and not necessarily in all of the virulent strains of *H. pseudoalbidus* in culture. The involvement of a plant factor in the pathogenicity of the fungus would also explain why *H. pseudoalbidus* is not pathogenic to the Japanese Ash (*Fraxinus mandshurica*), and why some individuals of *F. excelsior* are relatively resistant.

The interaction of **4** with specific targets in endophytic microorganisms of the European ash is also possible, similar to the recently reported interference of karrikins with bacterial quorum-sensing systems,[29] a mechanism by which endophytes could function as mediators between the plant and its pathogen. In summary, our findings are relevant, because they lay the ground for investigating the mode of action of this virulence factor of *H. pseudoalbidus* and for finding its molecular target. In addition, further research for understanding resistance mechanisms in *F. mandshurica* and individual *F. excelsior* trees is now possible. The identification of 3,4-dimethylpentan-4-olide as a virulence factor of *H. pseudoalbidus* will hopefully pave the way for control of a pathogen that currently threatens the entire population of *Fraxinus excelsior* in Europe.

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**Keywords:** ash dieback · *Fraxinus excelsior* · *Hymenoscyphus pseudoalbidus* · lactones · plant pathogens

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